Developmental Biology **248,** 40–51 (2002)
doi:10.1006/dbio.2002.0705

The Competence of Marginal Zone Cells to Become Spemann's Organizer Is Controlled by *Xcad2*

Vered Levy, Karen Marom, Sharon Zins, Natalia Koutsia, Ronit Yelin, and Abraham Fainsod[1]

Department of Cellular Biochemistry and Human Genetics, Hebrew University–Hadassah Medical School, P.O. Box 12272, Jerusalem 91120, Israel

The organizer in vertebrate embryos is responsible for the formation of the primary body axis. In amphibian embryos, the organizer forms in the dorsal marginal zone (prospective dorsal mesoderm) at a location determined by the point of sperm entry. Using inducible versions of axis-inducing proteins, it has been shown that, irrespective of the mode of secondary axis induction, organizer formation in the ventral marginal zone is temporally restricted from the midblastula transition to the onset of gastrulation. Here, we show that the competence of marginal zone cells to respond to organizer-inducing signals is under temporal control, one of the regulators being the homeobox transcription factor *Xcad2*. Overexpression of *Xcad2* restricts the temporal competence for axis induction, whereas partial loss of function expands this competence, supporting our suggestion. We propose that *Xcad2* competes with putative axis-inducing signals within the marginal zone to prevent expression of organizer-specific genes. Elimination of endogenous *Xcad2* allows for the activation of organizer genes beyond the normal competence window during early/mid-gastrulation. We conclude that *Xcad2*, through its early expression in the ventrolateral marginal zone, terminates the competence of this embryonic region to respond to organizer-inducing signals by preventing the activation of organizer-specific genes.

***Key Words: siamois*; axis induction; competence; transcription factor; *Xenopus*; caudal genes; *Cdx*; *gsc*; mesoderm.**

INTRODUCTION

The vertebrate body axis is established by the instructive effects of an "embryonic organizer." The existence of an organizer was first shown in the classical experiments of Spemann and Mangold (1924) using amphibian embryos. Following heterotopic transplantation of the dorsal blastopore lip into the ventral marginal zone (VMZ), an additional embryonic body axis was formed. Since then, dorsalization of the mesoderm and neural induction have been attributed to this embryonic structure (Harland and Gerhart, 1997). Recent work has identified genes with expression confined to the organizer region (reviewed by De Robertis, 1995; De Robertis *et al.*, 2000; Harland and Gerhart, 1997; Lemaire and Kodjabachian, 1996). When expressed in the VMZ, some of these genes can perform most of the organizer activities, while others perform only a limited repertoire. Among the genes that can induce secondary axes are elements of the early Wnt signaling pathway, such as *siamois* (Lemaire *et al.*, 1995), organizer-specific transcription factors such as *goosecoid* (gsc; Cho *et al.*, 1991), and organizer-secreted BMP antagonists such as *chordin* and *noggin* (Sasai *et al.*, 1994; Smith *et al.*, 1993).

Formation of the organizer initiates with activation of the early Wnt signaling pathway, which in turn activates the expression of organizer-specific genes (De Robertis *et al.*, 2000; Harland and Gerhart, 1997). Sperm entry, through a mechanism yet unknown, results in the early reorganization of the egg cytoplasm known as cortical rotation (Gerhart *et al.*, 1989). Cortical rotation leads to the dorsal accumulation of proteins involved in Wnt signaling, like dishevelled and β-catenin (Larabell *et al.*, 1997; Miller *et al.*, 1999; Schneider *et al.*, 1996), and subsequently, nuclear translocation of β-catenin in the future dorsal side of the embryo (De Robertis *et al.*, 2000; Harland and Gerhart, 1997; Heasman, 1997; Schohl and Fagotto, 2002). During the midblastula transition (MBT), with the onset of zygotic transcription, genes like *siamois* and *twin* are activated by β-catenin, resulting in the formation of Spemann's organizer (Brannon *et al.*, 1997; Carnac *et al.*, 1996; De Robertis *et al.*, 2000; Fan *et al.*, 1998; Laurent *et al.*, 1997).

[1] To whom correspondence should be addressed. Fax: +972-2-6415848. E-mail: fainsod@cc.huji.ac.il.

0012-1606/02 $35.00

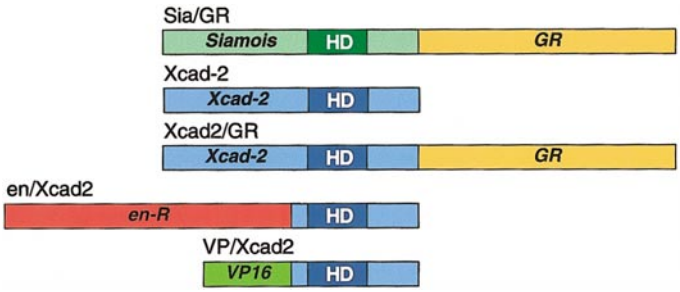

FIG. 1. Chimeric proteins. Schematic representation of the different *Xcad2* and *siamois* constructs utilized. The *Xcad2* and *siamois* proteins were fused to the GR domain. The *Xcad2* homeodomain was fused to the *engrailed* repression domain or the *VP16* activation domain.

Recently, several groups have performed detailed temporal studies of the competence to induce secondary axes. Employing several inducible proteins, it could be shown that the induction of secondary axes can only be achieved up until early gastrulation (Darken and Wilson, 2001; Kodjabachian and Lemaire, 2001; Melby *et al.*, 1999; Shapira *et al.*, 2000). These studies relied on the use of downstream effectors of the Wnt pathway, like Xtcf-3 (Darken and Wilson, 2001) and siamois (Kodjabachian and Lemaire, 2001), generally believed to represent the Nieuwkoop center signal. Also, similar results were obtained with antimorph versions of *Xvex1* and *Xvent2*/*Vox*, both homeobox genes activated by BMP signaling that normally function as repressors of organizer-specific gene expression (Melby *et al.*, 1999; Shapira *et al.*, 2000). These observations show that formation of Spemann's organizer is restricted to blastula and early gastrula stages before the neural-inducing and mesodermal-patterning roles of the organizer are believed to take place.

Xcad2, a transcription factor of the *caudal* homeobox type, has also been linked to the formation of the organizer. Overexpression of *Xcad2* results in ventralized embryos (Epstein *et al.*, 1997), and this ventralization is independent of an active BMP signaling pathway (Pillemer *et al.*, 1998b). *Xcad2* downregulates the expression of organizer-specific genes, such as *gsc*, *Xnot2*, *Otx2*, *Xlim1*, and *XFKH1* (Pillemer *et al.*, 1998b). Like the ventralizing effect, downregulation of organizer expression is not mediated through BMP signaling (Pillemer *et al.*, 1998b). Taken together, *Xcad2* functions as an anti-organizer during early gastrulation independent of BMP signaling. The temporal and spatial patterns of *Xcad2* expression place its transcripts in the nonorganizer marginal zone at the onset of gastrulation (Pillemer *et al.*, 1998a) in agreement with this proposed role.

The observation that *Xcad2* can antagonize both the dorsalizing and axis-inducing effects of blocking the BMP signaling pathway (Pillemer *et al.*, 1998b) further supports the suggestion that the ventralizing effect of *Xcad2* is exerted in a BMP-independent manner. Here, we show that, while induction of a secondary axis in *Xenopus* embryos is restricted to the developmental period between MBT and

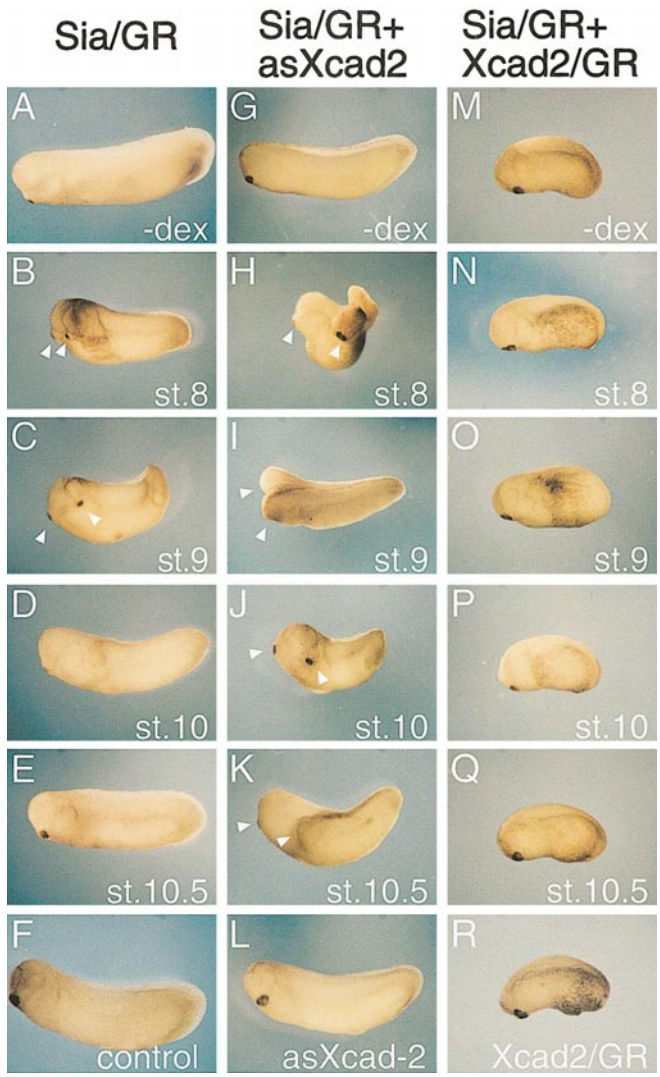

FIG. 2. *Xcad2* modifies the competence of the mesoderm to differentiate into Spemann's organizer. (A–E) Embryos injected ventrally with sia/GR mRNA and treated with dex at the stages stated exhibit a high incidence of secondary axis induction (arrowheads) as a result of activation at blastula stages. (F) Control uninjected sibling embryo. (G–K). Embryos coinjected ventrally with sia/GR and antisense *Xcad2* RNA. The constructs were activated at the stages as marked and exhibit secondary axis induction also during gastrula activation. (L) Injection of antisense *Xcad2* RNA does not result in secondary axis induction. (M–Q) Ventral coinjection of sia/GR with Xcad2/GR and activation at different developmental stages. This combination results in very weak secondary axis induction at all stages tested. (R) Ventral injection of Xcad2/GR mRNA and activation at stage 8 result in a weak ventralization.

the onset of gastrulation, the response of MZ cells to organizer-inducing signals is controlled by the activity of the homeodomain protein Xcad2. *Xcad2* accomplishes the change in competence of the VMZ by preventing the possible activation of organizer genes. The expression of *Xcad2* in the ventrolateral MZ therefore restricts the repertoire of genes that can be expressed by these cells, thereby changing their developmental competence.

MATERIALS AND METHODS

Chimeric Protein Constructs

Chimeric proteins were produced by amplifying specific protein domains and fusing them with the right adaptors to ensure in-frame continuity. All fusion proteins were sequenced to ensure the absence of PCR-induced mutations. In the case of the sia/GR and Xcad2/GR, the full-length *Xenopus* proteins were fused to the GR domain. The GR domain used included amino acids 512–777 of the human glucocorticoid receptor (Kolm and Sive, 1995). The obligatory activator or repressor versions of Xcad2 include amino acids 173–302 of the *Xcad2* protein product fused to the *Drosophila* engrailed repressor domain (Jaynes and O'Farrell, 1991) or the *HSV VP16* activation domain (Friedman *et al.*, 1988) as described for *Xvex1* (Shapira *et al.*, 2000).

RT-PCR Analysis

Embryos injected and treated as described were processed for RNA extraction. RT-PCRs were carried out in the exponential phase of the amplification curve as described (Shapira *et al.*, 2000). Primers used were: *histone H4*- forward CGGGATAACATTCAGGGTATCACT, reverse ATCCATGGCGGTAACTGTCTTCCT for 18 cycles at 55°C; *gsc*- forward AGGCACAGGACCCATCTTCAC, reverse CCCTTTTAACCTCTTCGTCCGC for 24 cycles at 54°C; *ADMP*- forward GAGCTGCAGCTTGATGAG, reverse GCCACAGTCCAGAGGTTA for 24 cycles at 60°C; *chordin*- forward ATTCATCCCTTCCAACAC, reverse CACACTCACATACGCAGAC for 24 cycles at 53°C; *BMP4*- forward TGTGAGGAGTTTCCATCACG, reverse TTGTTCTTCTTCCTGGACCG for 22 cycles at 57°C; *siamois*- forward TCAGAACAGAAGAGCCAG, reverse AGTGTGGTGATTCAAGAAG for 29 cycles at 52°C.

Embryo and Explant Manipulation

Xenopus embryos were obtained by *in vitro* fertilization and incubated in 0.1× MBSH. For microinjection, two-cell embryos were transferred to 1× MBSH and subsequently returned to 0.1× MBSH for continued incubation.

Activation of proteins fused to the hormone binding domain of the *glucocorticoid receptor* was performed by addition of dexamethasone (Sigma) to the incubation medium at a final concentration of 4 μg/ml at the specified stages. Inhibition of protein synthesis was achieved by incubation in cycloheximide (10 μg/ml; Sigma). When necessary, the dexamethasone treatment was performed 30 min after the treatment with cycloheximide was initiated.

Ventral and dorsal marginal zone explants were dissected at the onset of gastrulation when the dorsal lip becomes apparent. The marginal zone explants were incubated in 0.3× MBSH until the desired developmental stage as determined on sibling embryos.

RESULTS

Xcad2 Controls the Competence of VMZ Cells to Respond to Axis-Inducing Signals

Previous results in our laboratory have shown that *Xcad2* can function as a ventral gene by opposing the formation of secondary axes, preventing the expression of organizer-specific genes, and inducing the ventralization of *Xenopus* embryos when overexpressed. All these activities can be achieved in the absence of BMP signaling (Pillemer *et al.*, 1998b). These observations, together with the temporal restriction of axis induction in the VMZ and the ventrolateral pattern of expression of *Xcad2* during early gastrulation, led to the hypothesis that *Xcad2* might negatively regulate the induction of secondary organizers in the VMZ. In order to test whether *Xcad2* expression is responsible for a change in the competence of the VMZ to respond to organizer-inducing signals, we studied the axis-inducing capacity of *siamois* in the VMZ concomitant with *Xcad2* overexpression or partial loss-of-function. An inducible siamois protein was constructed by fusing the hormone binding domain of the human *glucocorticoid receptor* (GR) to its carboxy terminus (Fig. 1) (Kolm and Sive, 1995). The resulting chimeric protein is inactive until exposed to dexamethasone (dex). Embryos were injected with mRNA encoding the sia/GR fusion protein and treated with dex at different developmental stages (Figs. 2 and 3). Ventral *siamois* activity during blastula stages up to the onset of gastrulation results in the efficient induction of secondary axes (Figs. 2B, 2C, and 3A). Activation of sia/GR from early gastrulation onwards did not result in twinned embryos (Figs. 2D, 2E, and 3A). The use of *in situ* hybridization to identify the secondary axes gave the same temporal window for secondary axis induction (not shown). These results are in complete agreement with the published temporal analysis of secondary axis induction (Kodjabachian and Lemaire, 2001).

Coinjection of *Xcad2* antisense RNA together with the sia/GR mRNA led to an extension in the competence time window (Figs. 2H–2K), resulting in efficient secondary axis induction also at stages 10.5 and 11 (33 and 21%, respectively; Fig. 3A). At these stages, sia/GR alone was shown to be unable to induce secondary organizer activity (Figs. 2B–2E and 3A). Similar results were obtained irrespective of whether the embryos were injected at the 2- to 4-cell stage (Fig. 3A) or in the C4 blastomere at the 32-cell stage (not shown). At gastrula stages, *siamois* can induce anterior head structures only when the endogenous *Xcad2* activity is reduced (Figs. 2J and 2K). *Xcad2* antisense RNA injection on its own results in dorsalized embryos with short trunks and enlarged heads (Fig. 4D) (Epstein *et al.*, 1997), and this phenotype can be rescued by overexpression of wild type *Xcad2* (Fig. 4F). These results support the conclusion that *Xcad2* plays an inhibitory role on the capacity of *siamois* to induce secondary axes in the VMZ. Together, these observations suggest that an early *Xcad2* function is to terminate

the competence of cells outside the DMZ region to form an organizer.

Xcad2 Antimorph Acts Like Antisense Xcad2 in Extending the Competence Window

An *Xcad2* antimorph, in a manner similar to partial loss of *Xcad2* activity, was expected to cause an expansion of the temporal competence for organizer formation. To test this prediction, the *Xcad2* homeodomain was fused to the *VP16* activation domain (VP/Xcad2) or the *engrailed* repression domain (en/Xcad2; Fig. 1). RNA encoding either the obligatory activator or repressor proteins was injected into embryos and compared with *Xcad2* overexpression. The VP/Xcad2 protein gave a phenotype similar to the wild-type Xcad2 protein (not shown), resulting in ventralized embryos with a dorsoanterior index (DAI) of about 2 (Epstein *et al.*, 1997; Kao and Elinson, 1988). Ectopic expression of the obligatory repressor construct, en/Xcad2, dorsalized embryos to a DAI of about 7.5 (Fig. 4C). The effect of the en/Xcad2 protein can be rescued by overexpression of *Xcad2* (Fig. 4E), suggesting that, like antisense *Xcad2* RNA (Figs. 4D and 4F), it targets the *Xcad2* activity in the embryo. Further characterization of these constructs was performed by studying their effect on the *Xcad2* downstream gene, *Xpo* (Pillemer *et al.*, 1998b; Sato and Sargent, 1991). en/Xcad2 mRNA injection, like antisense *Xcad2* RNA, downregulates *Xpo* expression (Figs. 4I and 4J). These results identify en/Xcad2 as the antimorph version with a transcriptional activity opposite to the Xcad2 wild type protein.

In order to test the effect of the *Xcad2* antimorph on secondary axis induction, en/Xcad2-encoding RNA was coinjected together with sia/GR mRNA to determine whether it can change the temporal competence for secondary axis induction. In the presence of en/Xcad2, *siamois* can efficiently induce the formation of secondary axes through midgastrulation in a manner similar to antisense *Xcad2* RNA (Fig. 3A). These results suggest that antagonism or reduction of the endogenous *Xcad2* activity extends the competence window of ventrolateral marginal zone cells to respond to organizer-inducing signals.

Xcad2 Can Block the Formation of Endogenous or Ectopic Organizers

The competence-modifying effect of *Xcad2* was studied as a function of time by using an inducible Xcad2 protein, constructed by fusing the full open reading frame to the GR domain (Xcad2/GR; Fig. 1). To test its effect on ectopic organizers, Xcad2/GR was coinjected with sia/GR. Dexamethasone addition activated both constructs, the axis inducer, *siamois*, and the organizer antagonist, *Xcad2*, simultaneously. Under these conditions, Xcad2 strongly reduced the axis-inducing activity of *siamois*, even when activated during blastula stages 8 and 9 (Figs. 2N, 2O, and 3A). The very weak secondary axes obtained lacked any recognizable organization.

Injection of increasing amounts of mRNA encoding the inducible Xcad2 protein, together with a constant amount of sia/GR RNA and activation of the proteins at stage 8.5, shows that the effect of *Xcad2* is concentration-dependent (Fig. 3B). While injection of low amounts of *Xcad2* sense RNA has a slight effect on the efficiency of axis induction by *siamois*, high amounts can efficiently block the formation of secondary axes. These results support a possible antagonism between *Xcad2* and organizer induction.

The temporal effect of *Xcad2* on the endogenous organizer was tested by ectopically expressing Xcad2/GR in the dorsal region of the embryo, and activating it by dex addition at different stages, from MBT to midgastrulation. At each time point, the DAI of the embryos was determined (Fig. 5). When activated during blastula stages 8 or 9, the ectopic Xcad2/GR inhibited the formation of the endogenous organizer. Among the injected embryos, approximately 10% of them had very mild axial defects (DAI 4–5). The remainder of the embryos were distributed between two equal-sized groups, one with severe organizer defects and no apparent axis (DAI 0–1), and the other with anterior defects (DAI 2–3). Dorsal Xcad2/GR activation from the onset of gastrulation onwards results in a decrease in the fraction of embryos with severe organizer defects and an increase in the fraction of very mildly affected embryos (Fig. 5). The fraction of embryos with anterior truncations remained constant at all stages of activation, and they represent the posteriorizing effect of *Xcad2*, which takes place during gastrulation (Epstein *et al.*, 1997; R.Y. and A.F., unpublished observations). Embryos with a DAI of 0–1 suggest that *Xcad2* can exert its antagonistic effect on the endogenous organizer as well as on ectopic ones. Furthermore, the effect of *Xcad2* on the endogenous organizer is restricted in time to the same stages during which we have shown that *Xcad2* affects secondary axis formation, and it is the same time window during which organizer formation can occur.

Xcad2 Prevents the Activation of Organizer-Specific Genes

Analysis of the expression of organizer-specific genes provided molecular evidence of a temporal window of competence to respond to organizer-inducing signals. Embryos injected ventrally with sia/GR mRNA were treated with dex at different developmental stages, and the expression of *gsc*, *chordin* (Sasai *et al.*, 1994), and *anti-dorsalizing morphogenetic protein* (ADMP; Moos *et al.*, 1995) was analyzed by RT-PCR (Shapira *et al.*, 2000). All three organizer-specific genes showed increased transcript levels, as compared with control embryos, only when the siamois protein was activated prior to gastrulation (Fig. 6A). A similar analysis of the ventral gene, *BMP-4* (Fainsod *et al.*, 1994), revealed that the sia/GR protein can downregulate its expression only when activated during blastula stages (Fig. 6A). *Siamois* was unable to activate organizer-specific gene expression in VMZ explants from the onset of gastrulation (Fig. 6B). These results suggest that *siamois* is unable to induce secondary axes in the VMZ during gastrulation

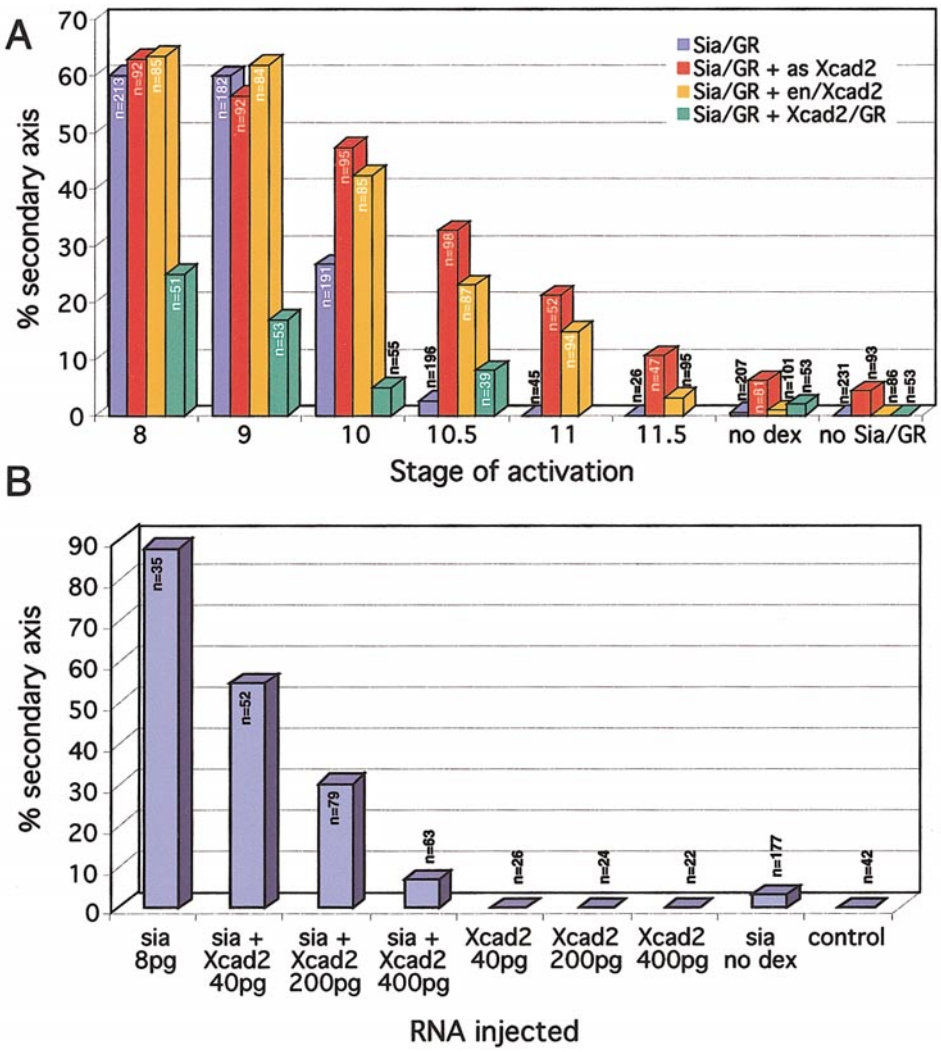

FIG. 3. *Xcad2* activity regulates organizer induction during gastrulation in the VMZ. (A) Embryos were injected ventrally with sia/GR mRNA (4 pg) alone or in combination with antisense *Xcad2* (1 ng), en/Xcad2 (160 pg), or Xcad2/*GR* (400 pg) RNA. The GR constructs were activated at different developmental stages and the fraction of embryos with secondary axes determined. (B) Injection with a constant amount of sia/GR RNA (8 pg) alone or with increasing amounts of Xcad2/GR mRNA (40, 200, and 400 pg). The fraction of secondary axes induced was scored.

due to a loss in its ability to activate the transcription of organizer-specific genes.

Ectopic *Xcad2* has been shown to efficiently downregulate the expression of organizer-specific genes, while antisense *Xcad2* RNA injected radially into embryos seems to expand the organizer region (Pillemer *et al.*, 1998b). Importantly, neither antisense nor antimorph *Xcad2* is able to induce secondary axes (Fig. 3A). In our system, the temporal pattern of expression of *goosecoid*, *chordin*, and *ADMP*, compared with control embryos, remained unchanged in antisense *Xcad2*-injected embryos (Fig. 6C). However, all three genes achieved higher expression levels than in control embryos (Fig. 6C). *BMP-4* transcription is reduced in embryos injected with antisense *Xcad2* RNA (Fig. 6C), in agreement with the proposed role of *Xcad2* as a required activator of *BMP-4* expression (Pillemer *et al.*, 1998b). Under these conditions, the organizer inducer, *siamois*, was unaffected. We conclude that endogenous *Xcad2* functions by restricting and/or preventing the expression of organizer genes along the ventrolateral marginal zone. This restriction becomes evident with the onset of gastrulation as a result of the normal accumulation of Xcad2 protein and can account for the loss of axis-forming competence of the ventrolateral MZ. Ventral expression of *siamois* concomitant with a reduction in *Xcad2* levels should therefore allow the activation of organizer-

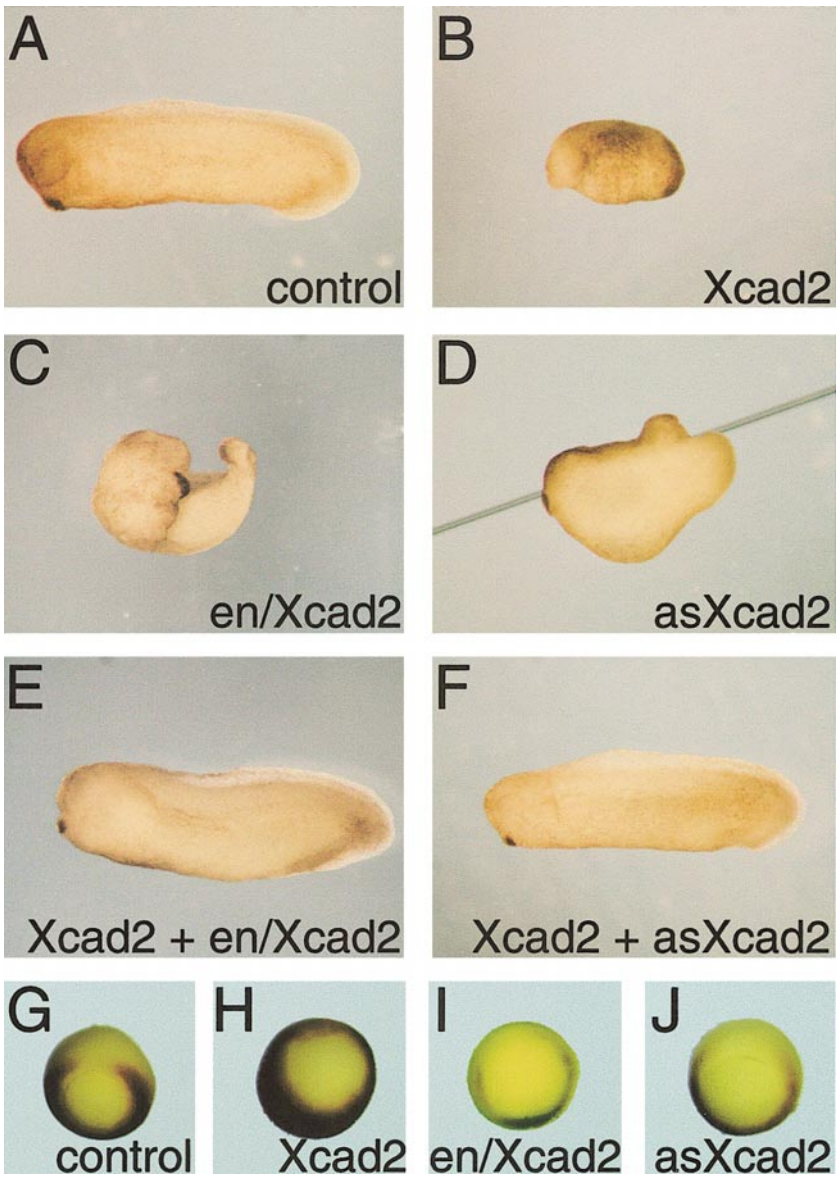

FIG. 4. Specificity of the *Xcad2* antisense and antimorph constructs. (A–F) Phenotypic effects of overexpression of the different *Xcad2* constructs. (A) Control embryo. (B) *Xcad2* overexpression results in ventralized embryos. (C) Radial overexpression of the en/Xcad2 protein results in an antimorphic phenotype opposite to that of *Xcad2* overexpression. (D) Radial injection of antisense *Xcad2* RNA results in dorsalized embryos. (E) Coinjection of *Xcad2* RNA and en/Xcad2 mRNA results in the phenotypic rescue of the effects of both constructs. (F) Injection of *Xcad2* mRNA together with antisense *Xcad2* RNA rescues the phenotypes of the embryos. (G–J) The regulatory influence of the *Xcad2* constructs on the ventrolateral downstream gene, *Xpo*. (G) Control *Xpo* expression pattern. (H) *Xcad2* overexpression causes the expansion of *Xpo* expression. Radial injection of either en/Xcad2 mRNA (I) or antisense *Xcad2* RNA (J) downregulate *Xpo* expression similarly.

specific genes beyond the competence window. Ventral coinjection of sia/GR and antisense *Xcad2* RNA or Xcad2 antimorph results in activation of *goosecoid*, *chordin*, and *ADMP* expression at all stages studied (Figs. 6B and 6D). Our results suggest a model where *Xcad2* competes for the regulation of organizer genes that are important for axis induction.

Direct Competition between Xcad2 and Siamois for Organizer Gene Expression

The availability of GR proteins allowed us to study whether inhibition of organizer-specific gene expression is mediated by the Xcad2 protein or requires the synthesis of downstream targets. Embryos coinjected with the sia/GR

and Xcad2/GR mRNAs accumulated these proteins in inactive form. At MBT, protein synthesis was inhibited in these embryos by cycloheximide treatment, and 30 min afterwards, the inducible proteins were activated with dex. Early protein synthesis inhibition resulted in the elimination of endogenous *gsc* expression (Fig. 7C), probably due to the absence of its endogenous activators such as *siamois*. Under the same conditions, the injected sia/GR protein can ectopically activate *gsc* transcription (Fig. 7D). The Xcad2/GR protein in the presence of cycloheximide and dex can prevent the ectopic activation of *gsc* by sia/GR (Fig. 7L). These results further support the competence-modifying activity of *Xcad2* and show that this activity relies on the Xcad2 protein and not on downstream targets.

DISCUSSION

A number of assays have been developed to study the organizer phenomenon in vertebrate embryos. In *Xenopus*, the induction of a secondary body axis by ectopic gene expression in the VMZ is an important functional assay (Harland and Gerhart, 1997). The axis induction assay relies on the inherent competence of cells in the ventral and lateral MZ to respond to organizer-inducing signals like their dorsal counterparts. Since fertilization can take place anywhere throughout the animal cap, the entire marginal zone/prospective mesodermal ring should be capable of differentiating as an organizer. Ventral zygotic genes like *Xwnt-8* (Christian *et al.,* 1991) raise a problem in light of the competence of the VMZ to differentiate as Spemann's organizer. If the competence to respond to organizer-inducing signals is maintained during gastrulation, the embryo would be dorsalized by the zygotic expression of genes like *Xwnt-8*, as demonstrated by the injection of its mRNA (Smith and Harland, 1991; Sokol *et al.*, 1991). We conclude that this competence is temporally restricted, to allow signals with organizer-inducing potential to function in additional developmental processes. Taking advantage of inducible, secondary axis-forming proteins, we studied the temporal regulation of organizer induction. Our results show that the competence is under temporal control, limiting the developmental period during which an organizer can be induced, and thus preventing the induction of supernumerary organizers.

Temporal Regulation of Spemann's Organizer Induction

Recently, temporal analysis of the competence for secondary axis induction has been performed with inducible versions of the *Xvex-1* antimorph (Shapira *et al.,* 2000), *Xvent-2*/*Vox* antimorph (Melby *et al.*, 1999), activated *XTcf-3* (Darken and Wilson, 2001), and *siamois* (Kodjabachian and Lemaire, 2001; this work). All these constructs show that, irrespective of the molecular pathway activated, secondary axis induction exhibits a clear temporal competence that ends with the onset of gastrulation. All axis-inducing constructs require the responding tissue, the marginal zone, to remain competent to differentiate into an organizer-type tissue, thus suggesting that the capacity of the marginal zone to differentiate along this pathway is under temporal control.

Experimentally, the VMZ plays the role of the responding tissue due to its inherent capacity to respond to axis (organizer)-inducing signals (Harland and Gerhart, 1997). This capacity is in agreement with the whole marginal zone initially being competent to differentiate as Spemann's organizer. On the other hand, reusing signaling pathways for alternative developmental processes would require a change of differentiation competence. The post-MBT ventralizing effect of *Xwnt-8* (Christian and Moon, 1993; Hamilton *et al.,* 2001) and the secondary axis induction resulting from the injection of its RNA (Smith and Harland, 1991; Sokol *et al.*, 1991) both function by signaling through the canonical Wnt pathway (Darken and Wilson, 2001). The competence of the marginal zone to respond to organizer-inducing signals must therefore terminate with the activation of *Xwnt-8* at gastrulation to prevent the formation of ectopic organizers or dorsalization of the embryo.

The competence to induce secondary axes may also be related to the capacity of the MZ cells to become mesoderm. The temporal competence for mesoderm induction was previously determined by using animal caps as the responding tissue, and was mapped up to midgastrulation (stage 11; Jones and Woodland, 1987; Steinbach *et al.*, 1997). This time window differs from the temporal competence to induce ectopic organizers using dex-dependent axis-inducing constructs (stage 10–10.5). We conclude that the different competence windows define organizer formation and mesoderm induction as separate specification decisions, not necessarily independent, as suggested by their distinct temporal controls (Harland and Gerhart, 1997).

Xcad2 As a Marginal Zone Competence Modifier

The loss of secondary axis-inducing activity as a function of time raised the question concerning the mechanism controlling this change in competence. Assuming the involvement of a ventrolateral signal at the onset of gastrulation that affects the marginal zone and modifies its competence, the homeobox gene *Xcad2* was studied. This gene exhibits a BMP-independent ventralizing activity (Pillemer *et al.,* 1998b). It is expressed in the ventrolateral marginal zone at the onset of gastrulation and its transcripts are excluded from the organizer region (Pillemer *et al.,* 1998a). Reduction of the endogenous *Xcad2* activity either by antisense RNA injection or via the Xcad2 antimorph, prolonged the competence of the ventrolateral MZ to respond to organizer-inducing signals until mid/late gastrulation, identifying this gene as a central component in terminating the competence to respond to axis-inducing signals. The temporal and spatial pattern of *Xcad2* expression is therefore in complete agreement with its proposed

competence-modifying activity, as shown by the loss-of-function approaches.

The mode of action of *Xcad2* in the context of organizer formation is of particular interest. *Xcad2* overexpression downregulates all organizer genes tested, including *goosecoid*, *chordin*, *Xlim1*, *Otx2*, *XFKH1*, *Xnot2*, and *ADMP* (Pillemer *et al.*, 1998b). Partial loss-of-function or antagonism of the *Xcad2* activity allows the expansion of the domains of dorsal gene expression (Pillemer *et al.*, 1998b). The *Xcad2* organizer inhibitory activity can be achieved in the absence of BMP signaling; furthermore, *Xcad2* overexpression also efficiently blocks the axis-inducing and dorsalizing activity that results from interfering with BMP signaling (Harland and Gerhart, 1997; Pillemer *et al.*, 1998b). Importantly, loss of *Xcad2* activity does not result either in secondary axis induction or in ectopic sites of organizer gene expression. On the other hand, reducing *Xcad2* activity allowed *siamois* to induce organizer-specific gene expression and secondary axes during gastrulation. Also, there is an inverse relationship between the axis-inducing activity of *siamois* and the level of *Xcad2* activity, and even the endogenous organizer is sensitive to *Xcad2* activity in a time-dependent manner, with this sensitivity being strongest prior to gastrulation. Finally, the inhibitory effect of *Xcad2* on the axis-inducing activity of *siamois* does not require the activation of downstream genes. Taken together, these observations suggest a model where *Xcad2* prevents the expression of downstream targets of organizer-inducing factors like *siamois*.

The results presented here identify *Xcad2* primarily by loss-of-function approaches, as a negative regulator of organizer formation. *Xcad2* is normally expressed only in the lateral and ventral MZ during late blastula and early gastrula stages. This pattern of expression, together with the organizer-antagonizing activity of *Xcad2* suggest two repressive functions during organizer establishment. The first role involves spatial restriction of the organizer region. This function is supported by the expansion of the expression domains of organizer genes in embryos with reduced *Xcad2* activity (Pillemer *et al.*, 1998b). The second and more important role involves modification of the competence of the nonorganizer MZ to respond to Nieuwkoop center-like signals. Significantly, the temporal and spatial patterns of *Xcad2* expression emphasize that the competence modifying activity is achieved in cells that would never under normal circumstances express organizer-specific genes, but these same cells will express *Xwnt-8*, for example, which signals through the same Wnt signaling pathway active in organizer formation (Darken and Wilson, 2001; Hamilton *et al.*, 2001). *Xcad2* would therefore prevent ventral and lateral MZ cells from becoming an organizer if they ever receive such a signal during gastrulation. This change in competence allows the activation of signaling pathways and genes with axis-inducing potential in the ventrolateral MZ without detrimental effects. Interestingly, in several instances, *caudal* homeobox genes have been shown to be under Wnt regulation (Ikeya and Takada, 2001; Lickert *et al.*, 2000). Therefore, a model could be suggested where *Xwnt-8* upregulates the gene(s) that would prevent its own detrimental effects, i.e., secondary axis induction or dorsalization.

The analysis of the competence of the marginal zone to differentiate as a Spemann's organizer serves as a model system to study specific steps in the progression along a differentiation pathway. In the present study, the competence of the MZ is defined functionally as the capacity to respond to organizer-inducing signals. Our study defines a novel step along a differentiation pathway defined by a loss in the ability to express a specific group of genes, not yet expressed, thus restricting the differentiation potential.

Negative Regulation of Organizer Formation

Establishment of the organizer-inducing signal in *Xenopus* begins with the fertilization event that activates the Wnt signaling pathway through cortical rotation (De Robertis *et al.*, 2000; Heasman, 1997). Activation of the signaling pathway leading to organizer formation is evident from the dorsal accumulation of disheveled and β-catenin, two elements of the Wnt signaling pathway (Larabell *et al.*, 1997; Miller *et al.*, 1999; Schneider *et al.*, 1996). Dorsal localization of the organizer is in part the result of localized activation of the inducing signal. On the other hand, the change in the competence of the responding tissue suggests a negative regulatory mechanism. The negative regulation prevents the formation of supernumerary organizers that could result from activation of additional Wnt signals during subsequent morphogenetic events.

The *Xcad2* activity described is different from the anti-organizer activity normally attributed to BMP signaling. Although both exhibit antagonism to the organizer, there is maternal BMP activity (Hemmati-Brivanlou and Thomsen, 1995), and targets of the BMP pathway (*Xvex-1* and *Xvent-2*) are efficiently activated soon after MBT (Schohl and Fagotto, 2002; Shapira *et al.*, 1999). The same BMP targets once converted into antimorphs, become efficient axis inducers on their own (Melby *et al.*, 1999; Onichtchouk *et al.*, 1998; Shapira *et al.*, 2000). BMP signaling establishes a network of repressors of organizer gene expression that functions as an inhibitory threshold for organizer formation (Melby *et al.*, 1999; Shapira *et al.*, 2000). In contrast, *Xcad2* activity appears toward the onset of gastrulation (Pillemer *et al.*, 1998a). Our results show that *Xcad2* blocks the potential activation of organizer-specific genes within its expression domain. The *Xcad2* protein, like other *caudal* proteins, has been identified as a transcriptional activator. It may therefore interact with cofactors that can modify its transcriptional effect. Apparently, it does not require the activation of downstream targets that function as transcriptional repressors. Independent of its mode of action, *Xcad2* activity is essential to modify the VMZ competence.

The zygotic phase of organizer formation begins with the activation of genes like *siamois* by the nuclearly localized β-catenin (Carnac *et al.*, 1996). Together with the results

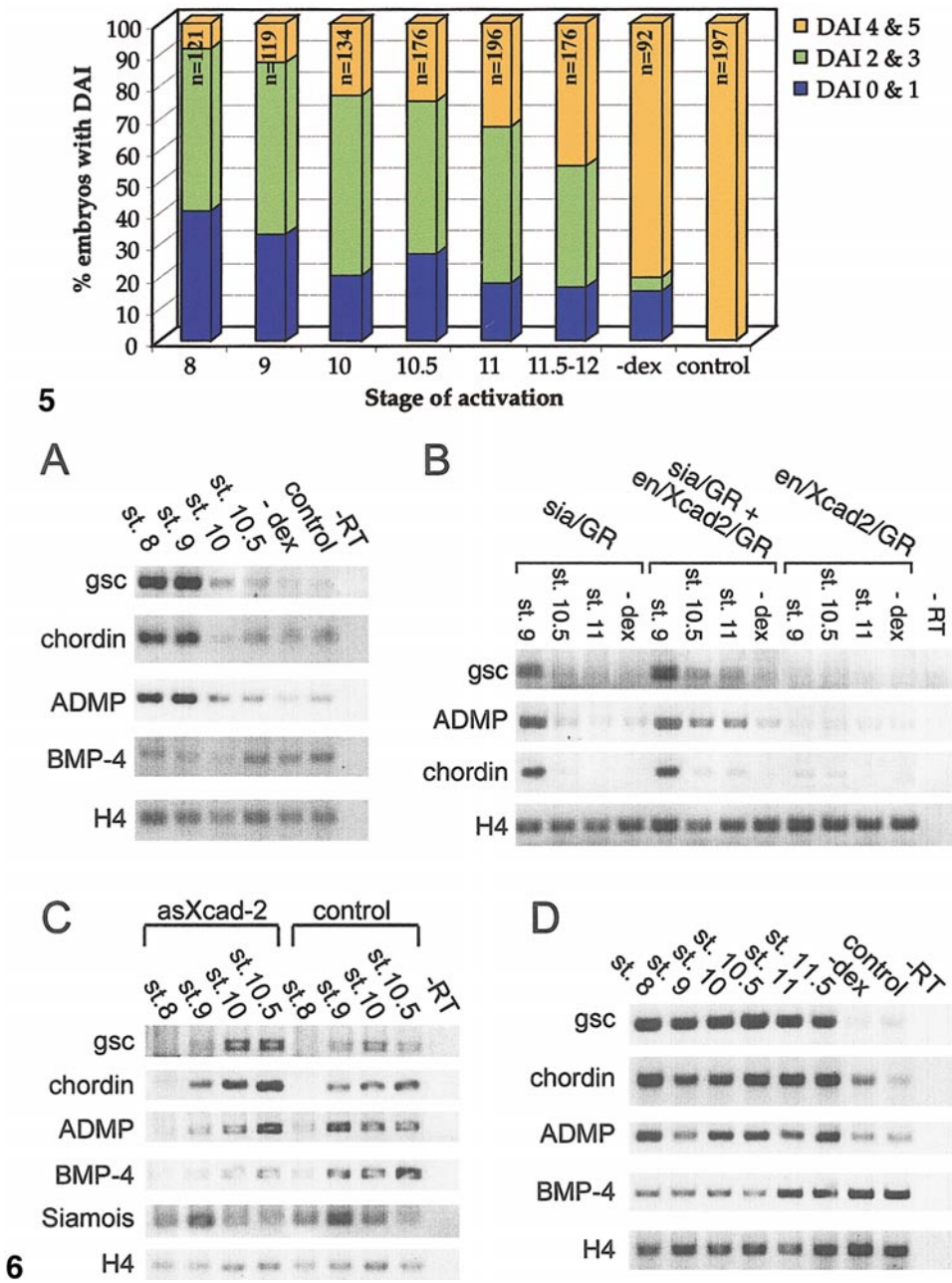

FIG. 5. Early dorsal *Xcad2* expression represses the formation of the endogenous organizer. Embryos were injected dorsally with 480 pg of Xcad2/GR mRNA and treated with dex at different developmental stages. At each time point, the embryos were divided into very mild axial defects (DAI 4–5), moderate anterior effects (DAI 2–3), and strong inhibition of organizer activity (DAI 0–1). The graph represents the compilation of a number of experiments where the percentage of each type of embryos is represented.

FIG. 6. Expression of organizer-specific genes during gastrulation depends on the competition between *siamois* and *Xcad2*. (A) Activation of organizer-specific genes by *siamois* is also restricted to blastula stages. RT-PCR of RNA samples prepared at stage 11 from embryos injected with sia/GR and treated with dex between stages 8 and 10.5. The genes tested were *gsc*, *chordin*, *ADMP*, *BMP-4*, and *histone H4* as a loading control. (B) The activity of *siamois* is restricted in explanted VMZ. Embryos were injected with sia/GR, en/Xcad2/GR, or a combination of both RNAs. VMZ explants were prepared and treated with dex at the specified stages. RNA extraction was performed at about stage 12. (C) *Xcad2* normally downregulates the expression of organizer-specific genes during gastrulation. Antisense *Xcad2* RNA was injected radially into four-cell embryos. At the specified stages, injected and control embryos were processed for RNA extraction and RT-PCR analysis under identical conditions. (D) Reduction of *Xcad2* function allows *siamois* to activate organizer-specific genes during gastrulation. Sia/GR and *Xcad2* antisense RNAs were ventrally coinjected. Activation by dex was performed at the stages specified, and RNA extraction was performed at about stage 12 to all samples together. In all experiments, the control samples are from uninjected embryos.

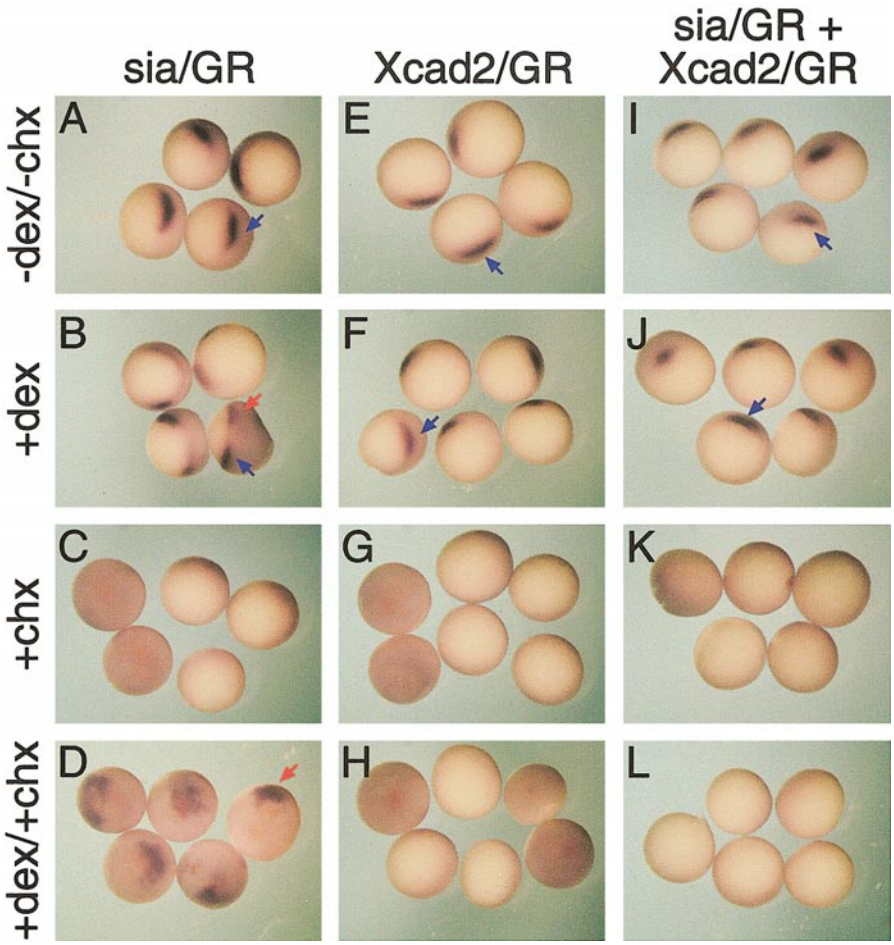

FIG. 7. Inhibition of secondary axis induction by Xcad2 can take place in the absence of protein synthesis. Embryos were injected with sia/GR and Xcad2/GR mRNAs singly (A–H) or in combination (I–L) and treated with dexamethasone (dex) and cycloheximide (chx). At the onset of gastrulation, the embryos were analyzed for changes in the expression of the organizer-specific gene, *gsc*. The blue arrows mark the endogenous *gsc* expression; the red arrows point to the ectopic *gsc* expression.

presented here, three mechanisms (1 positive, 2 negative) have been identified that work in concert to ensure the formation of a single organizer in the dorsal side of the vertebrate embryo. First, the organizer-inducing signal (Wnt signal) is localized to only one side of the embryo (Harland and Gerhart, 1997). Second, from MBT onwards, downstream genes of the BMP signaling pathway, like *Xvex-1*, establish an inhibitory threshold limiting the formation of an organizer to the site of highest Wnt signal (Shapira *et al.*, 2000). Third, the competence to form an organizer is terminated along the ventrolateral MZ by *Xcad2*. Together, these mechanisms ensure that such a crucial decision as the formation of the organizer is under spatial and temporal regulation. The model suggested illustrates how signaling pathways or genes can be used repeatedly for various developmental decisions without mistakenly inducing embryonic malformations.

ACKNOWLEDGMENTS

We thank Martin Blum, Herbert Steinbeisser, and Rolando Rivera-Pomar for support and discussions throughout the course of the work and their comments regarding the manuscript. We thank Dale Frank, Ralph Rupp, Eddy De Robertis, Marc Kirschner, David Kimelman, Sergei Sokol, Christof Niehrs, and Hazel Sive for plasmids. We are thankful to Aharon Razin, Graciela Pillemer, and Ayala Frumkin for critically reading the manuscript. This work was supported in part by grants of The Israel Science Foundation founded by The Israel Academy of Sciences and Humanities and Volkswagen Stiftung (to A.F.).

REFERENCES

Brannon, M., Gomperts, M., Sumoy, L., Moon, R. T., and Kimelman, D. (1997). A β-catenin/XTcf-3 complex binds to the *siamois*

promoter to regulate dorsal axis specification in *Xenopus*. *Genes Dev*. **11,** 2359–2370.

Carnac, G., Kodjabachian, L., Gurdon, J. B., and Lemaire, P. (1996). The homeobox gene *Siamois* is a target of the Wnt dorsalisation pathway and triggers organiser activity in the absence of mesoderm. *Development* **122,** 3055–3065.

Cho, K. W. Y., Blumberg, B., Steinbeisser, H., and De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: The role of the *Xenopus* homeobox gene *goosecoid*. *Cell* **67,** 1111–1120.

Christian, J. L., McMahon, J. A., McMahon, A. P., and Moon, R. T. (1991). Xwnt-8, a *Xenopus Wnt-1/int-1*-related gene responsive to mesoderm inducing growth factors, may play a role in ventral mesoderm patterning during embryogenesis. *Development* **111,** 1045–1055.

Christian, J. L., and Moon, R. T. (1993). Interactions between *Xwnt-8* and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of *Xenopus*. *Genes Dev*. **7,** 13–28.

Darken, R. S., and Wilson, P. A. (2001). Axis induction by wnt signaling: Target promoter responsiveness regulates competence. *Dev. Biol*. **234,** 42–54.

De Robertis, E. M. (1995). Dismantling the organizer. *Nature* **374,** 407–408.

De Robertis, E. M., Larraín, J., Oelgeschläger, M., and Wessely, O. (2000). The establishment of Spemann's organizer and patterning of the vertebrate embryo. *Nat. Rev. Genet*. **1,** 171–181.

Epstein, M., Pillemer, G., Yelin, R., Yisraeli, J. K., and Fainsod, A. (1997). Patterning of the embryo along the anterior-posterior axis: The role of the *caudal* genes. *Development* **124,** 3805–3814.

Fainsod, A., Steinbeisser, H., and De Robertis, E. M. (1994). On the function of *BMP-4* in patterning the marginal zone of the *Xenopus* embryo. *EMBO J.* **13,** 5015–5025.

Fan, M. J., Gruning, W., Walz, G., and Sokol, S. Y. (1998). Wnt signaling and transcriptional control of Siamois in Xenopus embryos. *Proc. Natl. Acad. Sci. USA* **95,** 5626–5631.

Friedman, A. D., Triezenberg, S. J., and McKnight, S. L. (1988). Expression of a truncated viral trans-activator selectively impedes lytic infection by its cognate virus. *Nature* **335,** 452–454.

Gerhart, J., Danilchik, M., Doniach, T., Roberts, S., Rowning, B., and Stewart, R. (1989). Cortical rotation of the *Xenopus* egg: Consequences for the anteroposterior pattern of embryonic dorsal development. *Development* **107**(Suppl.), 37–51.

Hamilton, F. S., Wheeler, G. N., and Hoppler, S. (2001). Difference in XTcf-3 dependency accounts for change in response to beta-catenin-mediated Wnt signalling in Xenopus blastula. *Development* **128,** 2063–2073.

Harland, R., and Gerhart, J. (1997). Formation and function of Spemann's organizer. *Annu. Rev. Cell Dev. Biol*. **13,** 611–667.

Heasman, J. (1997). Patterning the *Xenopus* blastula. *Development* **124,** 4179–4191.

Hemmati-Brivanlou, A., and Thomsen, G. H. (1995). Ventral mesodermal patterning in *Xenopus* embryos: Expression patterns and activities of *BMP-2* and *BMP-4*. *Dev. Genet*. **17,** 78–89.

Ikeya, M., and Takada, S. (2001). Wnt-3a is required for somite specification along the anteroposterior axis of the mouse embryo and for regulation of cdx-1 expression. *Mech. Dev*. **103,** 27–33.

Jaynes, J. B., and O'Farrell, P. H. (1991). Active repression of transcription by the engrailed homeodomain protein. *EMBO J.* **10,** 1427–1433.

Jones, E. A., and Woodland, H. R. (1987). The development of the animal cap cells in *Xenopus*: A measure of the start of animal cap competence to form mesoderm. *Development* **101,** 557–563.

Kao, R. K., and Elinson, R. P. (1988). The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. *Dev. Biol*. **127,** 64–77.

Kodjabachian, L., and Lemaire, P. (2001). Siamois functions in the early blastula to induce Spemann's organiser. *Mech. Dev*. **108,** 71–79.

Kolm, P. J., and Sive, H. L. (1995). Efficient hormone-inducible protein function in *Xenopus laevis*. *Dev. Biol*. **171,** 267–272.

Larabell, C. A., Torres, M., Rowning, B. A., Yost, C., Miller, J. R., Wu, M., Kimelman, D., and Moon, R. T. (1997). Establishment of the dorso-ventral axis in Xenopus embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. *J. Cell Biol*. **136,** 1123–1136.

Laurent, M. N., Blitz, I. L., Hashimoto, C., Rothbacher, U., and Cho, K. W. (1997). The Xenopus homeobox gene twin mediates Wnt induction of goosecoid in establishment of Spemann's organizer. *Development* **123,** 4905–4916.

Lemaire, P., Garret, N., and Gurdon, J. B. (1995). Expression cloning of *siamois*, a *Xenopus* homeobox gene expressed in dorsal vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* **81,** 85–94.

Lemaire, P., and Kodjabachian, L. (1996). The vertebrate organizer: structure and molecules. *Trends Genet*. **12,** 525–531.

Lickert, H., Domon, C., Huls, G., Wehrle, C., Duluc, I., Clevers, H., Meyer, B. I., Freund, J. N., and Kemler, R. (2000). Wnt/(beta)-catenin signaling regulates the expression of the homeobox gene Cdx1 in embryonic intestine. *Development* **127,** 3805–3813.

Melby, A. E., Clements, W. K., and Kimelman, D. (1999). Regulation of dorsal gene expression in Xenopus by the ventralizing homeodomain gene Vox. *Dev. Biol*. **211,** 293–305.

Miller, J. R., Rowning, B. A., Larabell, C. A., Yang-Snyder, J. A., Bates, R. L., and Moon, R. T. (1999). Establishment of the dorsal–ventral axis in Xenopus embryos coincides with the dorsal enrichment of dishevelled that is dependent on cortical rotation. *J. Cell Biol*. **146,** 427–437.

Moos, M. J., Wang, S., and Krinks, M. (1995). Anti-dorsalizing morphogenetic protein is a novel TGF-β homolog expressed in the Spemann organizer. *Development* **121,** 4293–4301.

Onichtchouk, D., Glinka, A., and Niehrs, C. (1998). Requirement for *Xvent-1* and *Xvent-2* gene function in dorsoventral patterning of *Xenopus* mesoderm. *Development* **125,** 1447–1456.

Pillemer, G., Epstein, M., Blumberg, B., Yisraeli, J. K., De Robertis, E. M., Steinbeisser, H., and Fainsod, A. (1998a). Nested expression and sequential downregulation of the *Xenopus caudal* genes along the anterior–posterior axis. *Mech. Dev*. **71,** 193–196.

Pillemer, G., Yelin, R., Epstein, M., Gont, L., Frumkin, Y., Yisraeli, J. K., Steinbeisser, H., and Fainsod, A. (1998b). The *Xcad-2* gene can provide a ventral signal independent of *BMP-4*. *Mech. Dev*. **74,** 133–143.

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K., and De Robertis, E. M. (1994). Xenopus *chordin*: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79,** 779–790.

Sato, S. M., and Sargent, T. D. (1991). Localized and inducible expression of *Xenopus-posterior* (*Xpo*), a novel gene active in early frog embryos, encoding a protein with a "CCHC" finger domain. *Development* **112,** 747–753.

Schneider, S., Steinbeisser, H., Warga, R. M., and Hausen, P. (1996). Beta-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos. *Mech. Dev.* **57,** 191–198.

Schohl, A., and Fagotto, F. (2002). beta-catenin, MAPK and Smad signaling during early Xenopus development. *Development* **129,** 37–52.

Shapira, E., Marom, K., Levy, V., Yelin, R., and Fainsod, A. (2000). The Xvex-1 antimorph reveals the temporal competence for organizer formation and an early role for ventral homeobox genes. *Mech. Dev.* **90,** 77–87.

Shapira, E., Marom, K., Yelin, R., Levy, A., and Fainsod, A. (1999). A role for the homeobox gene *Xvex-1* as part of the *BMP-4* ventral signaling pathway. *Mech. Dev.* **86,** 99–111.

Smith, W. C., and Harland, R. M. (1991). Injected Xwnt-8 RNA acts early in Xenopus embryos to promote formation of a vegetal dorsalizing center. *Cell* **67,** 753–765.

Smith, W. C., Knecht, A. K., Wu, M., and Harland, R. M. (1993). Secreted *noggin* protein mimics the Spemann organizer in dorsalizing *Xenopus* mesoderm. *Nature* **361,** 547–549.

Sokol, S., Christian, J. L., Moon, R. T., and Melton, D. A. (1991). Injected Wnt RNA induces a complete body axis in Xenopus embryos. *Cell* **67,** 741–752.

Spemann, H., and Mangold, H. (1924). Uber Induktion von Embryonalanlagen durch Implantation Artfremder Organisatoren. *Roux's Arch. Entw. Mech.* **100,** 599–638.

Steinbach, O. C., Wolffe, A. P., and Rupp, R. A. (1997). Somatic linker histones cause loss of mesodermal competence in Xenopus. *Nature* **389,** 395–399.

Received for publication November 7, 2001
Revised April 18, 2002
Accepted April 19, 2002
Published online June 25, 2002